Properties of a Halophil Nicotinamide–Adenine Dinucleotide Phosphate-Specific Isocitrate Dehydrogenase

PRELIMINARY STUDIES OF THE SALT RELATIONS AND KINETICS OF THE CRUDE ENZYME

By D. M. AITKEN, A. J. WICKEN AND A. D. BROWN

School of Microbiology, University of New South Wales, Kensington, Sydney, N.S.W. 2033, Australia

(Received 21 July 1969)

The effects of chlorides on NADP-specific isocitrate dehydrogenase from *Halobacterium salinarum* were investigated. The enzyme is stabilized by potassium chloride and sodium chloride and this effect is discussed in relation to the Hill (1913) equation. Kinetics of the enzyme were studied within a range of concentrations of potassium chloride and sodium chloride. Apparent Michaelis constants for both substrates were affected by salt concentration, the effect being greater in sodium chloride than in potassium chloride. Minimal apparent Michaelis constants for both substrates were similar to the corresponding constants reported for yeast isocitrate dehydrogenase. $V_{\max}$ was maximal in each salt at a concentration of about 1M. The maximum was higher in sodium chloride than in potassium chloride. At salt concentrations above about 2.3M, the apparent $V_{\max}$ was lower in sodium chloride than in potassium chloride, and at salt concentrations below 0.75–1.0M, each salt behaved as a linear activator of the enzyme. Within this concentration range salt and NADP⁺ acted competitively; the activation by salt was overcome at finite concentrations of NADP⁺. At concentrations above about 1M, potassium chloride was a linear non-competitive inhibitor of the enzyme. Within the range 1.0–2.5M, sodium chloride was also a linear non-competitive inhibitor, but above 2.5M it caused more pronounced inhibition.

All halophilic bacterial enzymes so far described have salt requirements or tolerances greater than those of corresponding enzymes from other types of organism (Larsen, 1962, 1967; Brown, 1964; Hochstein & Dalton, 1968; Aitken & Brown, 1969). There is, however, little detailed information, either kinetic or physicochemical, about the mechanism of interaction between salt and any halophil enzyme. Moreover, there are differences in the salt relations of some individual enzymes. For example, isocitrate dehydrogenase from *Halobacterium salinarum* was reported by Baxter & Gibbons (1956) to have an optimum sodium chloride requirement of about 1.2M and a potassium chloride optimum in the range 2–4M. Larsen (1962) gave the salt optimum of the corresponding enzyme from *Sarcina morrhuae* as 0.8M; Hubbard & Miller (1968) reported the optimum for an isocitrate dehydrogenase from *H. cutirubrum* to be about 0.5M-sodium chloride. Aitken & Brown (1969) found an apparent optimum of about 2M-sodium chloride or -potassium chloride for this enzyme in a non-pigmented strain of *H. salinarum* and observed that activity of the enzyme was low at concentrations of sodium chloride or sodium chloride + potassium chloride high enough to support growth

of the organism (3–5M). Although there are differences in the apparent salt optima of halophil isocitrate dehydrogenases reported by different authors, there is general agreement that the salt optimum of the enzyme is below the salt concentration at which the organisms can survive. The total intracellular salt concentration of viable halophilic bacteria is normally at least as great as that of their growth medium, although the intracellular K⁺/Na⁺ ratio can be very much larger than the corresponding extracellular ratio (Christian & Waltho, 1962).

The present paper describes the first part of an investigation of the kinetics and salt relations of a halophil isocitrate dehydrogenase.

METHODS AND MATERIALS

Organism. A colourless strain of *H. salinarum* was obtained from Professor H. Larsen of Trondheim, Norway. This is the strain used for the detection of all the enzymes of the tricarboxylic acid cycle and for a preliminary assessment of the salt requirements of the isocitrate dehydrogenase (Aitken & Brown, 1969). In all respects except pigmentation, the organism appears to be typically halophilic. The organism was grown in 1-litre batches with

rotary agitation for 6 days at 30°C (to late exponential phase) in the salts solution described by Sehgal & Gibbons (1960) containing Oxoid peptone (1%, w/v).

Measurement of pH. A Radiometer TTT1 pH-meter fitted with a GK 202-C combined electrode was used. No correction for Na⁺ error was necessary in the pH range used.

Reagents. Sodium DL-isocitrate, sodium *threo*-D₅-(+)-isocitrate and NADP⁺ (monosodium salt) were reagent-grade preparations of the Sigma Chemical Co., St Louis, Mo., U.S.A., and were used without further purification. Salts and buffer constituents were reagent-grade chemicals from various commercial sources and were used without further purification.

Enzyme preparations and assay. The organisms were harvested by centrifugation, washed once at 0°C in 5M-NaCl containing 0.02M-MgCl₂ and resuspended in 5M-NaCl at 0°C; the yield of organisms from 1 litre of culture was suspended in 10 ml of 5M-NaCl. The chilled suspension was disrupted ultrasonically for 1.5 min with an MSE ultrasonic disintegrator (60 W at 18 000–20 000 Hz). The disrupted suspension was centrifuged at 19 000g for 15 min at 0°C to remove whole cells and debris. The supernatant fluid was then centrifuged at 27 000g for 45 min at 0°C. The straw-coloured supernatant solution was used as the crude enzyme preparation. This solution was stored at –10°C, at which temperature it remained unfrozen. The isocitrate dehydrogenase activity remained constant for at least 2 weeks under these conditions; enzyme assays were made up to 10 days after preparation of the extract.

Isocitrate dehydrogenase was assayed at 30°C by measuring the rate of formation of NADPH from NADP⁺ added in the presence of isocitrate. The standard enzyme assay solution contained (final concentrations): tris-HCl buffer (33 mM, final pH 8.35); MgCl₂ (3.3 mM); NADP⁺ (1 mM); sodium DL-isocitrate (4 mM); enzyme (0.033 ml, 330–990 μg of protein); salt as specified. The total volume was 1 ml. All assays were performed at least in duplicate; means of replicates were used as single points in subsequent statistical analyses. For kinetic studies the concentrations of NADP⁺ and isocitrate were varied as specified. Isocitrate was added before the enzyme and the reaction mixture, without NADP⁺, was incubated for 3 min at 30°C before the reaction was started by the addition of NADP⁺. Preliminary assays had shown that enzyme activity increased with preincubation for up to 3 min and thereafter remained constant up to 10 min (total). There was no detectable change in isocitrate concentration for up to 10 min in the absence of added NADP⁺. Unlike the NAD-specific isocitrate dehydrogenase of *Neurospora crassa* (Sanwal, Zink & Stachow, 1964), the enzyme was not activated by AMP. Incorporation of 1 mM-cyanide into the reaction mixture had no effect on the reaction rate. There was no measurable reoxidation of NADPH by the enzyme preparation. The formation of NADPH was measured at 340 nm in a Zeiss PMQ II spectrophotometer fitted with a recorder. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951).

Preliminary conventional double-reciprocal plots were drawn to confirm linearity, after which kinetic results were fitted statistically to the equation:

$$v = VA/(K + A) \quad (1)$$

with the Fortran computer programme 'Hyper' (Cleland, 1967) on an IBM 360 computer. Secondary plots, against salt concentration (or its reciprocal), of slopes and intercepts derived from eqn. (1) were fitted by a weighted-least-squares method with a computer programme obtained from Dr J. F. Morrison. For these calculations the concentration of added DL-isocitrate was halved to obtain the concentration of the biologically active dextrorotatory (*threo*-D₅) isomer (Vickery, 1962). Kinetic measurements with pure *threo*-D₅-(+)-isocitrate confirmed that the proportion of active isomer in the DL-preparation was exactly half.

RESULTS

pH relations of the crude enzyme. A preliminary determination of the pH relations of the enzyme was made within the nominal range pH 6.2–8.9 with sodium acetate-acetic acid, sodium phosphate and tris-hydrochloric acid buffers, all at 33 mM. The type and concentration of salt included in the assay solution affected the observed pH, the difference from the nominal pH being greatest with phosphate and least with tris. Within the range pH 6.3–7.9, 4M-sodium chloride lowered the observed pH of phosphate buffer by 1.1–1.2 pH units, and 4M-potassium chloride lowered the observed value by 0.3–0.5 pH unit. Within the range pH 7.9–8.7 4M-sodium chloride lowered the observed pH value of 33 mM-tris buffer by 0.1 pH unit whereas 4M-potassium chloride increased the observed value by 0.2 pH unit. The observed value, measured after addition of salt, was used in this work. Maximal activity of the enzyme occurred in tris buffer at pH 8.1–8.5, in which range any errors introduced by effects of salt on pH would be small. Fig. 1 shows salt and pH relations of the enzyme within a narrow pH range in tris buffers.

Effects of univalent cations. Fig. 2 shows a comparison of the effects of the chlorides of four univalent cations on activity of the enzyme. The relative abilities of the salts to support isocitrate dehydrogenase activity are dependent on salt concentration.

Effects of salt on stability of the enzyme. In order to express quantitatively the stabilization of the enzyme by salt, rates of inactivation were measured in the lower ranges of salt concentration. For this purpose the enzyme was incubated at 0°C in solutions of sodium chloride or potassium chloride buffered with 33 mM-tris-HCl to pH 8.0. At specified time-intervals samples were withdrawn and assayed immediately under standard conditions in the presence of the same salt at 1 M concentration. The results are plotted in Fig. 3 with the assumption that the relation between log (original activity/remaining activity) and time is linear. Secondary plots of log (first-order rate constant) from Fig. 3 (log *k*) against log [salt] are shown in Fig. 4. This is the method used by Baxter (1959) and by Holmes & Halvorson (1965) for

Fig. 1. (a) Effects of NaCl concentration on the pH relations of the enzyme in tris buffer (33 mM). Concentrations of NaCl were as follows; Δ , 0.5M; $\circ$, 1.0M; $\square$, 1.5M; $\bullet$, 2.0M; $\blacktriangle$, 3.0M; $\blacksquare$, 4.0M; $\blacktriangledown$, 5.0M. (b) Effects of KCl concentration on the pH relations of the enzyme in tris buffer (33 mM). Concentrations of KCl were as follows; $\square$, 0.5M; $\circ$, 1.0M; Δ , 2.0M; $\bullet$, 3.0M; $\blacksquare$, 4.0M.

Fig. 2. Effects of salts on activity of the enzyme. $\circ$, LiCl; $\square$, NaCl; Δ , KCl; $\bullet$, NH_4Cl .

presenting results of salt-stabilization studies of a halophil lactate dehydrogenase and malate dehydrogenase respectively.

Effects of salt on the kinetics of the enzyme. Apparent Michaelis constants are listed for a range of concentrations of sodium chloride and potassium chloride in Tables 1 and 2 respectively. The apparent $K_m(\text{isocitrate})$ was minimal at about 1.5M-potassium chloride, varied little between 1.5M- and 4.0M-potassium chloride and was consistently lower in potassium chloride than in sodium chloride. In sodium chloride the $K_m(\text{isocitrate})$ was minimal near

Fig. 3. (a) Stabilization of the enzyme by NaCl. The enzyme preparation was incubated at 0°C and then assayed at 30°C in 1M-NaCl. The lines from top to bottom represent rates of inactivation in the following concentrations of NaCl: $\circ$, 0.6M; $\square$, 0.8M; Δ , 1.0M; $\bullet$, 1.2M. (b) Stabilization of the enzyme by KCl. The enzyme preparation was incubated at 0°C and assayed at 30°C in 1M-KCl. The lines, from top to bottom, represent rates of inactivation in the following concentrations of KCl: $\circ$, 0.6M; $\square$, 0.8M; Δ , 1.0M; $\bullet$, 1.2M; $\blacksquare$, 1.4M; $\blacktriangle$, 1.75M.

0.75M and rose very sharply at lower salt concentrations. The apparent $K_m(\text{NADP})$ responded differently to the two salts. There was some evidence

Fig. 4. Linear regressions of $\log k$ (the first-order rate constant of enzyme inactivation, from Fig. 3) against concentration of (a) NaCl and (b) KCl.

Fig. 5. Effects of salt concentration on $V_{\max}$. In those parts of the relation depicted by a single common line, $V_{\max}$ was essentially a true $V_{\max}$, and the points are means of $V_{\max}$ values obtained by varying the concentration of each of the two substrates. The error bars indicate a mean standard error for six degrees of freedom. The concentration of the non-variable substrate was constant at all salt concentrations (see Table 1) and, at extreme salt concentrations, was evidently not saturating. Under these conditions $V_{\max}$ was different for each variable substrate and represented an apparent $V_{\max}$. Where this occurred the lines diverge and the error bars are standard errors for three degrees of freedom. $\circ$, Apparent $V_{\max}$ in NaCl, isocitrate concentration varying; Δ , apparent $V_{\max}$ in NaCl, NADP⁺ concentration varying; $\blacksquare$, apparent $V_{\max}$ in KCl, NADP⁺ concentration varying; $\blacktriangle$, apparent $V_{\max}$ in KCl, isocitrate concentration varying.

of a minimum in both 0.25M-sodium chloride and -potassium chloride. Below about 1M-salt the apparent K_m was lower in sodium chloride than in

Table 1. Apparent Michaelis constants of the enzyme in sodium chloride

Apparent Michaelis constants were determined with sodium DL-isocitrate as non-varying substrate at an initial concentration of 4mM (2mM for the biologically active isomer) or with NADP⁺ as non-varying substrate at an initial concentration of 1mM. Values are quoted $\pm$ S.E.M. for three degrees of freedom.

Concn. of NaCl (M)	$10^4 \times K_m$ (isocitrate) (M)	$10^4 \times K_m$ (NADP ⁺) (M)
0.15	4.28 ± 0.02	0.29 ± 0.05
0.25	2.32 ± 0.23	0.25 ± 0.03
0.50	1.40 ± 0.31	0.41 ± 0.13
0.75	1.18 ± 0.14	0.80 ± 0.09
1.0	1.30 ± 0.24	1.31 ± 0.22
1.5	1.48 ± 0.28	1.96 ± 0.58
2.0	1.67 ± 0.24	2.13 ± 0.30
2.5	1.70 ± 0.11	2.31 ± 0.43
3.0	2.36 ± 0.32	2.40 ± 0.36
4.0	3.77 ± 0.48	5.00 ± 0.55
5.0	4.36 ± 0.48	5.81 ± 0.15

Table 2. Apparent Michaelis constants of the enzyme in potassium chloride

The constants were determined and are quoted as in Table 1. NaCl (0.15M) was present in all assays involving KCl (see the text).

Concn. of KCl (M)	$10^4 \times K_m$ (isocitrate) (M)	$10^4 \times K_m$ (NADP ⁺) (M)
0.15	1.68 ± 0.26	0.46 ± 0.03
0.25	1.48 ± 0.02	0.38 ± 0.06
0.5	0.80 ± 0.09	0.77 ± 0.06
0.75	0.72 ± 0.08	0.99 ± 0.11
1.0	0.52 ± 0.11	1.23 ± 0.24
1.5	0.48 ± 0.06	1.36 ± 0.13
2.0	0.53 ± 0.08	1.60 ± 0.14
2.5	0.69 ± 0.18	1.84 ± 0.10
3.0	0.74 ± 0.18	2.02 ± 0.28
4.0	0.94 ± 0.12	2.33 ± 0.32

potassium chloride, but this relation was reversed above 1M-salt. The K_m (NADP) increased gradually in potassium chloride above 0.25M, but in sodium chloride there was a sharp increase above 3M-salt.

The response of $V_{\max}$ to changes in the concentration of each salt is shown in Fig. 5. Within the range 0.75–3.0M-sodium chloride and at concentrations of potassium chloride up to 3M values obtained for $V_{\max}$ were the same, whichever substrate was varied. At those salt concentrations therefore, the concentration of the fixed substrate was effectively saturating and values obtained for $V_{\max}$ can be treated as true $V_{\max}$ values. At high and low concentrations of sodium chloride and at high concentrations of potassium chloride, however, the $V_{\max}$

Fig. 6. (a) Double-reciprocal plots of kinetic results obtained by varying the isocitrate concentration in low (activating) concentrations of NaCl (upper figure) and of KCl (lower figure). The non-variable substrate, NADP⁺, was used at an initial concentration of 1 mM. The regressions were fitted statistically in all cases (see the text). In the upper figure the lines from top to bottom (right of intersection) were obtained with the following concentrations of NaCl: ○, 0.15 M; △, 0.25 M; □, 0.5 M; ●, 0.75 M. In the lower figure the lines correspondingly were obtained with the following concentrations of KCl: ○, 0.15 M; △, 0.25 M; □, 0.5 M; ●, 0.75 M; ▲, 1.0 M. (b) Double-reciprocal plots of kinetic results obtained by varying the NADP⁺ concentration in the presence of low (activating) concentrations of NaCl (upper figure) and of KCl (lower figure). The non-variable substrate, sodium DL-isocitrate, was used at an initial concentration equivalent to 2 mM of the biologically active isomer. In the upper figure the lines from top to bottom (right of intersection) were obtained with the following concentrations of NaCl: ○, 0.75 M; △, 0.5 M; □, 0.25 M. In the lower figure the lines correspondingly were obtained with the following concentrations of KCl: ○, 1.0 M; △, 0.75 M; □, 0.5 M; ●, 0.25 M. The regressions obtained with 0.15 M-NaCl and 0.15 M-KCl did not fit these patterns and have been omitted from the figure. In both parts of the figure, $1/v$ is expressed as $(\mu\text{mol/min per mg})^{-1}$.

was different for each variable substrate. These differences were associated with substantial increases in apparent Michaelis constants (Tables 1 and 2). They imply that at least one of the fixed substrates was at a less than saturating concentration and that values of $V_{\max}$ were apparent $V_{\max}$ values. Like the apparent Michaelis constants, $V_{\max}$ varied more in response to changes in sodium chloride concentration than it did to potassium chloride. This resulted in a reversal of the relation of the two series of $V_{\max}$ values at a concentration of about 2.25 M-salt.

The relations summarized in Tables 1 and 2 and Fig. 5 were dependent on the assay method inasmuch as activity below about 1 M-salt required substrate-stabilization of the enzyme. The enzyme was

completely inactivated if added before isocitrate to an assay mixture containing 0.15–0.25 M-salt. Above about 1.4 M-sodium chloride or about 1.8 M-potassium chloride there was no evidence of substrate-stabilization of the enzyme in this sense. Between 0.25 M- and about 1.4 M-salt there were intermediate degrees of substrate-stabilization under the conditions of assay. Dialysis against water caused complete inactivation of the crude enzyme. About 20% of the original activity was recovered by dialysis against 5 M-sodium chloride. Like the isocitrate dehydrogenase from pig heart (Moyle, 1956) the enzyme needed SO_4^{2-} to maintain full activity during dialysis of a partially purified preparation against 5 M-sodium chloride.

Double-reciprocal plots of the kinetic results for a

Fig. 7. (a) Double-reciprocal plots obtained by varying the isocitrate concentration in the presence of high (inhibitory) concentrations of NaCl (upper figure) and KCl (lower figure). The non-variable substrate, NADP^+ , was used at an initial concentration of 1 mM. In the upper figure the lines from top to bottom (right of intersection) were obtained with the following concentrations of NaCl: $\circ$, 5.0 M; Δ , 4.0 M; $\square$, 3.0 M; $\bullet$, 2.5 M; $\blacktriangle$, 2.0 M; $\blacksquare$, 1.5 M; ∇ , 1.0 M. In the lower figure the lines were correspondingly obtained with the following concentrations of KCl: $\circ$, 4.0 M; Δ , 3.0 M; $\square$, 2.5 M; $\bullet$, 2.0 M; $\blacktriangle$, 1.5 M. (b) As for (a) but with NADP^+ as variable substrate. The non-variable substrate, sodium DL-isocitrate, was used at an initial concentration equivalent to 2 mM of the active isomer. The upper and lower figures depict results obtained with NaCl and KCl respectively. The order of lines is the same as in (a) in both cases. In both parts of the figure $1/v$ is expressed as $(\mu\text{mol}/\text{min per mg})^{-1}$.

series of salt concentrations gave families of lines intersecting, in all but two cases, to the left of the ordinate (Figs. 6 and 7). The exceptional situation was encountered at low (activating) salt concentrations with NADP^+ as variable substrate, under which conditions the lines intersected to the right of the ordinate (Fig. 6b). Figs. 8 and 9 depict secondary plots of slopes and intercepts (from Figs. 6 and 7) against the reciprocal of salt concentration and salt concentration respectively.

DISCUSSION

The stabilization of an enzyme by a salt can have two implications which differ in time-scale. An enzyme might be stabilized over a long period against any of several forms of inactivation or there can be rapid effects of a change in salt concentration that are reflected in initial-velocity measurements. The reversibility of the two processes can be differ-

ent. This phenomenon is presumably true in principle for many enzymes but it becomes conspicuous at the salt concentrations used with halophilic organisms.

In the present work the effects of salt on stabilization of the enzyme for periods of up to 20 min at 0°C were investigated. The results were analysed and presented (Figs. 3 and 4) in the manner used by Baxter (1959) and by Holmes & Halvorson (1965). Both sets of authors stated that the slope of the secondary plot (as in Fig. 4 above) was numerically equal to the number of mol of salt required to stabilize 1 mol of enzyme against inactivation. Baxter (1959) found a value between 2 and 3 for sodium chloride and lactate dehydrogenase; Holmes & Halvorson (1965) derived a value of 4.5 for sodium chloride and malate dehydrogenase. The corresponding values for isocitrate dehydrogenase (Fig. 4) are 3.6 and 2.9 for sodium chloride and potassium chloride respectively.

Fig. 8. Secondary plots of slopes and intercepts from Fig. 6 against $1/[\text{Na}^+]$ and $1/[\text{K}^+]$ (b). The intercepts are the means of values obtained with both NADP^+ and isocitrate as variable substrates. This was not wholly justified at low concentrations of NaCl (a) when apparent V_{max} values were different for each variable substrate (Fig. 5). Secondary plots of individual intercept values however, were similar to the mean plot shown in (a). Mean values were plotted for simplicity and because this treatment was used primarily for qualitative purposes. With NADP^+ as variable substrate, secondary plots of slope were non-linear and have not been illustrated. The aberrant point in the plot of slope in (b) represents a value obtained in 0.15M-KCl at which concentration there were several possible sources of error (see the text). In both parts of the figure $1/v$ is expressed as $(\mu\text{mol/min per mg})^{-1}$.

This type of mathematical analysis is based on an equation proposed by Hill (1913) for the interaction of oxygen with haemoglobin. Some of the implications of the Hill equation were discussed by Atkinson (1966), who pointed out that the slope of a Hill plot (as in Fig. 4) is not a valid indication of the number of binding sites of a reactant (ligand) to a protein. Probably the only tentative conclusion to be drawn from Fig. 4 is that the enzyme requires at least 4 molecules of sodium chloride or at least 3 molecules of potassium chloride for stabilization under the experimental conditions. A precise conclusion requires more information than is available from the present analysis. These reservations apply also to the conclusions of Baxter (1959) and of Holmes & Halvorson (1965).

The effects of salts on the long-term stabilization of the isocitrate dehydrogenase were not studied in the present work, in which the enzyme was prepared and stored in $5\text{M-sodium chloride}$. The lowest concentration of sodium chloride used in the assay was 0.15M and this was present in all assays, including those made with salts other than sodium chloride. Sodium chloride additions were corrected for the

basal concentration (0.15M) and the final concentrations are as stated in the results. No difference was found between enzyme preparations stored in $4\text{M-potassium chloride}$ and $5\text{M-sodium chloride}$ when both were assayed under 'standard' conditions in $1\text{M-sodium chloride}$. No kinetic studies were made with the potassium chloride preparation.

Changes in the pH of a buffer caused by salts also become important at high salt concentrations. Holmes & Halvorson (1965) discussed this effect at some length, but then chose to ignore the shift in pH. They regarded the initial pH (i.e. the nominal value before the addition of salt) as valid. There seems to be no physicochemical justification for this decision, but Holmes & Halvorson (1965) did advance a biological one. This was that, when the nominal value of pH was used, a smooth curve was obtained through a series of buffers for the pH-activity relation of a halophil malate dehydrogenase. Conversely, our own results look better if presented with the observed pH. Arguments of this kind are difficult to sustain, however, since salt affects not only the observed pH of the buffer but also, presumably, the pH relations of an

Fig. 9. Secondary plots of slopes and intercepts from Fig. 7 against [NaCl] (a) and [KCl] (b). The plotted intercept values were means of values obtained by varying the concentrations of each of the substrates. For the reasons given with Fig. 8 this treatment was not wholly justified at high salt concentration. When individual values of intercepts were used, linear plots with slightly different slopes were obtained; their qualitative significance was not affected. In the secondary plots of slope, the upper regression ($\circ$) in each case is for NADP⁺ as variable substrate, the lower regression ($\square$) is for isocitrate. In both parts of the figure $1/v$ is expressed as $(\mu\text{mol/min per mg})^{-1}$.

enzyme. Salt is bound to change the pH of a buffer (cf. Robinson & Stokes, 1955) and the major uncertainty is whether or not any particular electrode assembly accurately measures the change. There seems to be no obvious reason why a glass electrode should not do this, if corrections for Na⁺ error are applied when necessary. If this assumption is wrong however, the error introduced by making it is small.

Figs. 2 and 5 show that any generalization about the order of effectiveness of the univalent cations in supporting activity of the enzyme must be qualified by the concentration of the salt. In this respect the isocitrate dehydrogenase differs from a halophilic soluble NADH-oxidizing system that, under the conditions of assay, had a high salt optimum (about 4M), and that was reported to show little change with salt concentration in the order of dependence on several univalent cations (Hochstein & Dalton, 1968).

According to Christian & Waltho (1962), the intracellular concentrations of K⁺ and Na⁺ in *H. salinarum* are about 4.6molal and 1.4molal respectively when the organism is grown in a medium containing 4M-sodium chloride and 0.03M-potassium chloride.

Our medium is only slightly different from this and similar intracellular concentrations of the two ions can be assumed to occur in our organism.

The minimal value for the apparent K_m (isocitrate) in potassium chloride was 48 μM ; in sodium chloride the minimum was 118 μM . The value in potassium chloride is similar to apparent constants reported for the corresponding enzyme from yeast [40 μM (Kornberg & Pricer, 1951); 58 μM (Bernofsky & Utter, 1966)] but greater than values published for the pig heart enzyme [2.6 μM (Moyle, 1956); 0.5 μM (Cleland & Thompson, 1965)]. The minimal apparent K_m (NADP) (25 μM) obtained in 0.25M-sodium chloride was similar to a value of 22 μM reported for the yeast enzyme by Bernofsky & Utter (1966).

Figs. 6 and 7 show that, except for expected similarities in the regions of salt optima, salt at all concentrations affected both the slopes and the intercepts of double-reciprocal plots of the kinetic results. There were irregularities associated with 0.15M-salt, when interpretation of results was complicated by pronounced isocitrate-stabilization of the enzyme and by the presence of 0.15M-sodium

chloride in all the assays made in the presence of potassium chloride.

Fig. 6 shows that at low concentrations both sodium chloride and potassium chloride acted as activators of isocitrate dehydrogenase. Since both salts affected slopes and intercepts of double-reciprocal plots, it follows (Cleland, 1963*b*) that each salt (or its cation) combines with the enzyme in such a way that its attachment is reversibly separated in the reaction sequence from the attachment of substrate to enzyme and that the net rate of attachment of the variable substrate to the enzyme is affected by the salt. With NADP⁺ as variable substrate, however, the effects of salt concentration were unusual inasmuch as the double-reciprocal plots intersected to the right of the ordinate. The consequence of this, in terms of kinetic constants, is that increasing salt concentration caused increases in both $V_{\max}$ and apparent K_m . A situation of this type is more commonly reflected in intersection of the primary plots to the left of the ordinate and below the abscissa (cf. Cleland, 1963*a,b*; Morrison, 1965). The present results can be regarded as a special case of competitive interaction in which the effect of salt is overcome at a finite subsaturating concentration of the variable substrate. Under this particular set of experimental conditions, salt and NADP⁺ each combine with the same form of the enzyme. Within the low (activating) range of salt concentration, therefore, there is a critical concentration of NADP⁺ at which reaction velocity is independent of salt concentration. This concentration was 0.19mM in sodium chloride and 0.44mM in potassium chloride.

Secondary plots of intercepts showed the activation by each salt to be linear (Fig. 8*b*). This was also true of the secondary plot of slope (Fig. 8*a*) with isocitrate but not with NADP⁺ as variable substrate. Numerical values can be calculated from the extrapolation of the secondary plots in Fig. 8 and represent kinetic constants for salt in the reaction (Cleland, 1963*b*). The identity of the constants cannot be determined, however, without detailed knowledge of the reaction mechanism.

Primary plots of results obtained in the high (inhibitory) range of salt concentration (Fig. 7) gave, with potassium chloride, single families of lines intersecting to the left of the ordinate. Secondary plots of slopes and intercepts (Fig. 9) showed that potassium chloride was a linear non-competitive inhibitor throughout the entire inhibitory concentration range.

In the inhibitory range of sodium chloride concentration, double-reciprocal plots for each variable substrate gave two families of lines intersecting at two points to the left of the ordinate (Fig. 7). The secondary plot of intercept from Fig. 7 against [sodium chloride] was linear throughout but the corresponding plot of slope was linear only between

1.0M- and 2.5M-sodium chloride (Fig. 9). Above 2.5M the secondary plot diverged sharply into what is possibly a second linear regression, but which is not depicted as such in Fig. 9 because the regression for that part of the concentration range was not fitted statistically. Kinetic constants can also be calculated by extrapolation of the linear regressions of Fig. 9, but, as previously, the meaning of these constants is uncertain until the reaction mechanism is understood.

Figs. 7 and 9 suggest that at high concentrations Na⁺, but not K⁺, causes a change in the state of the enzyme from that which exists at lower concentrations of salt (E_I) to a new state (E_{II}). The net rate of addition of each substrate to E_{II} is inhibited to a greater extent by sodium chloride than is the corresponding addition to E_I . This difference is reflected in the sharp rise in apparent K_m listed in Table 1. The change $E_I \rightleftharpoons E_{II}$ is reversed when both substrates are at saturating or near-saturating concentrations, a fact that is reflected in the linear plot of intercept against [sodium chloride] in Fig. 9*b*). This phenomenon is therefore another special case of competitive interaction, this time competitive inhibition, in which the reaction subject to competitive effects is a change in enzyme state.

Many of the results described above show that isocitrate dehydrogenase is more sensitive to Na⁺ than to K⁺. Thus, in the simplest terms, the enzyme is more active at optimum concentrations of Na⁺ than of K⁺ and is inhibited more by high concentrations of Na⁺ than of K⁺.

We thank Dr J. F. Morrison of the Biochemistry Department, Australian National University, for his interest, very valuable advice and the computer programmes referred to in the text.

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